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Award Number: DAMD17-03-1-0652

TITLE: Promoter Switching and Transcription Factor Usage During Breast Adipocyte Differentiation: Role in Aromatase Expression and Activity.

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REPORT DATE: September 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DAT	2. REPORT TYPE	3. DATES COVERED
01-09-2005 E	Final	1 Sept 2003 - 31 Aug 2005

4. TITLE AND SUBTITLE

Promoter Switching and Transcription Factor Usage During Breast Adipocyte Differentiation: Role in Aromatase Expression and Activity.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0652

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Kemmy M. Mizinga, Ph. D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Kansas City University of Medicine &
Biosciences
Kansas City, MO 64106-1453

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A murine cell-culture (3T3-L1) model of fibroblast-to-adipocyte differentiation was validated and used to demonstrate aromatase gene promoter-switching. Validation of a human cell culture model used in definitive further studies is nearly complete. The pace of progress was initially affected adversely by the loss of a technician who departed in February 2004 to relocate out of state with her family. Efforts to find a suitably qualified replacement were unsuccessful until late in October 2004. The new technician engaged to start work on November 29, 2004 was expected to greatly facilitate completion of work proposed under Specific Aim 1, and Aims 2 and 3 (corresponding to Tasks 1, 2 and 3, respectively) during the ensuing no-cost extension period. However, this technician had to be removed from the grant by the end of June 2005, for persistent substandard performance in execution and completion of essential project tasks. Despite significant personnel setbacks cited above, completion of ongoing work is projected for later in the last quarter of 2005.

15. SUBJECT TERMS
breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	10	

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Introduction

This project is based on evidence that in postmenopausal hormone-dependent breast cancer, *in situ* over-expression of aromatase (P450arom; the product of the CYP19 gene) by stromal cells results in increased local estrogen levels, which stimulate and/or support the proliferation of malignant breast epithelial cells (Green, 1990; Lippman et al., 1986). Conversely, estrogen deprivation induced by aromatase inhibitors is an effective treatment in some breast cancer patients (Goss and Strasser, 2001). Despite evidence supporting the importance of aromatase in initiation and development of breast cancer, knowledge about the regulation of aromatase expression is limited. Several distinct aromatase mRNA species have been found in normal vs. tumor adipose tissue, representing usage of alternate promoters in exon I (i.e., promoters I.3, II and I.4) (Agarwal et al., 1996; Zhou et al., 2001). Normal breast adipose tissue contains only low levels of aromatase and utilizes distal promoter I.4, whereas tumor adipose tissue expresses aromatase at higher levels and its mRNA is transcribed off proximal promoters II and I.3 (Zhou et al., 2001). Also, terminal differentiation of fibroblasts into mature adipocytes is accompanied by promoter-switching and greatly reduced aromatase expression. The underlying mechanism(s) of promoter switching are not well understood and the biological advantage(s), if any, conferred onto malignant cells remain undefined.

Body

A murine cell-culture (3T3-L1) model of fibroblast-to-adipocyte differentiation was validated and used to demonstrate aromatase gene promoter-switching. Validation of a human cell culture model to be used in further studies is nearly complete. The pace of progress was adversely affected by the loss of a technician who departed in February 2004 to relocate out of state with her family. Efforts to find a suitably qualified replacement were unsuccessful until October 2004. The new technician engaged to start work on November 29, 2004 was expected to greatly facilitate completion of work proposed under Specific Aim 1, and Aims 2 and 3 (corresponding to Tasks 1, 2 and 3, respectively) during the ensuing no-cost extension period. However, this technician had to be removed from the grant by the end of June 2005, for persistent substandard performance in execution and completion of essential project tasks. Despite significant personnel setbacks cited above, completion of ongoing work is projected for later in the last quarter of 2005.

Research accomplishments associated with each component of Tasks 1, 2 and 3 are presented below: [Task 1: To examine aromatase mRNA translational efficiency associated with aromatase exon I alternate promoters I.3, II and I.4. a) Amplify and quantify invariant region encoded by exons II through X to enable measurement of total aromatase mRNA levels by competitive RT-PCR. b) Amplify individual aromatase species using promoter-specific primer sets and quantify each species by competitive RT-PCR, and c) Determine promoter-specific aromatase mRNA distribution in monosomes vs. polysomes and establish translational efficiency associated with aromatase exon I alternate promoters I.3, II and I.4. Task 2: To determine stability and activity of the aromatase protein produced by exon I alternate promoters I.3, II and I.4. (Months 4-6): a) Quantify aromatase levels to evaluate effects of all stipulated test conditions and alternate promoter usage. b) Assess aromatase activity under stipulated test conditions and alternate promoter usage. Task 3. To determine transcription factor (TF) identity and activation patterns associated with terminal differentiation of breast fibroblast cells into adipocytes and with each of the three alternate exon I aromatase promoters (i.e., promoters I.3, II and I.4). a) Determine

transcriptional factor usage under all stipulated test conditions. b) Establish transcriptional factor usage under alternate promoter-usage patterns associated with undifferentiated and differentiated, and interim stages toward terminal breast adipocyte differentiation. c) Analyze, interpret and summarize all findings, and present data at national scientific conference. d) Prepare manuscript(s) for publication in scientific journal(s). e) Utilize findings to develop, and support submission of hypothesis-based research grant proposal application(s) to national funding agencies (e.g., NCI, CDMRP, etc.)]

Task 1a and 1b): Human aromatase cDNA mimics for subsequent use in quantitative RT-PCR were constructed using the method described by Callaci and Hentosh (1997). We slightly modified and improved the protocol. Briefly, a heterologous "neutral" double-stranded DNA fragment of known sequence (Clontech) was PCR amplified using a pair of forward and reverse "composite" primers. The composite forward primer contained at its 5'-end, three sets of gene-specific (i.e., aromatase) nucleotides and the 20-mer forward primer (5'-CGTGACCCTCCCCGCTATCT-3') for the "neutral" double-stranded DNA. Oligonucleotide forward primers specific for aromatase promoters I.4, I.3, or PII were those described by Harada et al., (1993) as follows: promoter I.4 = 5'-GACCAACTGGAGCCTG -3'; promoter I.3 = 5'-CCTTGTT TTGACTTGTAAC-3'; promoter PII= 5'-AACAGGAGCTATAGATG-3'. They were combined in the primer sequence: 5'-Promoter I.3-, PII-, and I.2-specific, followed by the 20-mer forward primer for the "neutral" double-stranded DNA (i.e., 5'-CCTTGTTTTGACTTGTAACAACAGGAGCTATAGATGGACCAACTGGAGCCTGCGTGA CC CTCCC CGCTATCT-3' The reverse primer for all the above mRNA species (and mimic) was derived from exon II: 5'-GTGCCCTCATAATTCCACAC-3'. Constructing one mimic incorporating all three primers specific for aromatase promoters I.4, I.3, and PII proved more expeditious than the originally proposed approach of making three separate constructs. Therefore this modified approach is being used in all subsequent experiments. A separate cDNA mimic was also constructed to amplify total aromatase transcripts, using the primer set described by Zhou et al., (2001) and "neutral" DNA specific nucleotides. All primers were synthesized and gel purified commercially (IDT Inc.) and subjected to one round of standard PCR using neutral DNA and composite primers. A second round of PCR was carried out using primers containing only aromatase-specific sequences. The resulting cDNA mimic was purified by centrifugation through Chromaspin® columns and then quantified by UV spectrophotometry.

Aromatase promoter usage was determined in three sets of 3T3-L1 cells: The first set of cells was untreated pre-confluent undifferentiated, the second was untreated spontaneously differentiated, and the third set was dexamethasone (DEX)-treated differentiated. A GenBank® search revealed that our human primers for promoters of interest (i.e., I.4, I.3 and PII) could be used in mouse cells. However, the reverse primer for the full human aromatase gene had insufficient homology with the mouse gene and could not be used in mouse cells. RNA was extracted from $5x10^6$ 3T3-L1 cells from each of the following three groups: preconfluent undifferentiated, spontaneously differentiated, and dexamethasone-induced differentiated cells. Briefly, cells were centrifuged for 10 min at 1100 rpm and the supernatant was aspirated. Cells were lysed using TRIzol (Gibco) and then chloroform was added to extract RNA, which was subsequently precipitated using isopropanol. RNA was washed with 75% ethanol and then pelleted and allowed to air-dry for 5-10 min. Thereafter, RNA was resuspended in nuclease-free water, allowed to incubate at 55-60°C for 10 min and then stored at -80°C while awaiting RT-

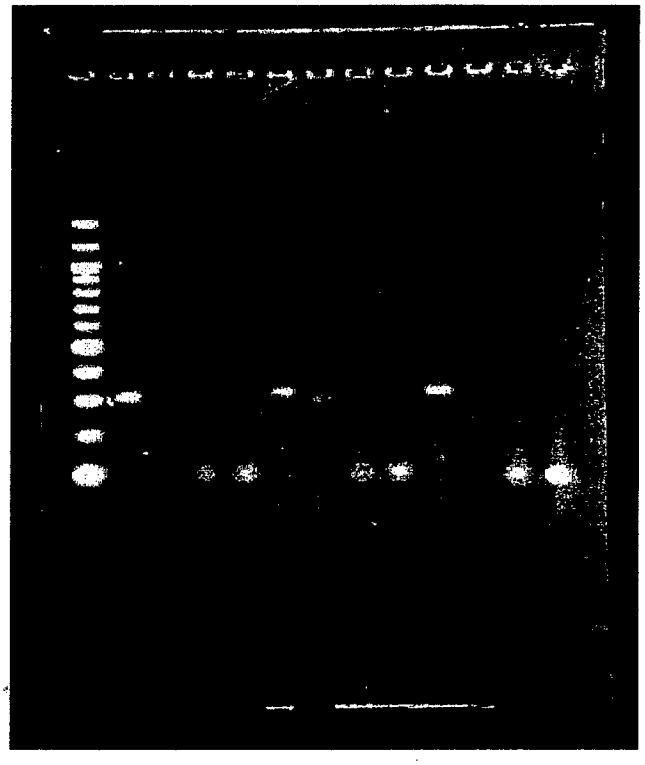


Figure 1:

Lane 1: 100 bp molecular weight marker

Lanes 2-5: Spontaneously differentiated 3T3-L1 cells
Lane 2: Promoter 1.3- specific aromatase mRNA
Lane 3: Promoter PII-specific aromatase mRNA
Lane 4: Promoter 1.4-specific aromatase mRNA
Lane 5: -RT

Lanes 6-9: Preconfluent undifferentiated 3T3-L1 cells
Lane 6: Promoter 1.3- specific aromatase mRNA
Lane 7: Promoter PII-specific aromatase mRNA
Lane 8: Promoter 1.4-specific aromatase mRNA
Lane 9: -RT

Lanes 10-13: DEX-treated differentiated 3T3-L1 cells
Lane 10: Promoter 1.3- specific aromatase mRNA
Lane 11: Promoter PII-specific aromatase mRNA
Lane 12: Promoter 1.4-specific aromatase mRNA
Lane 13: -RT

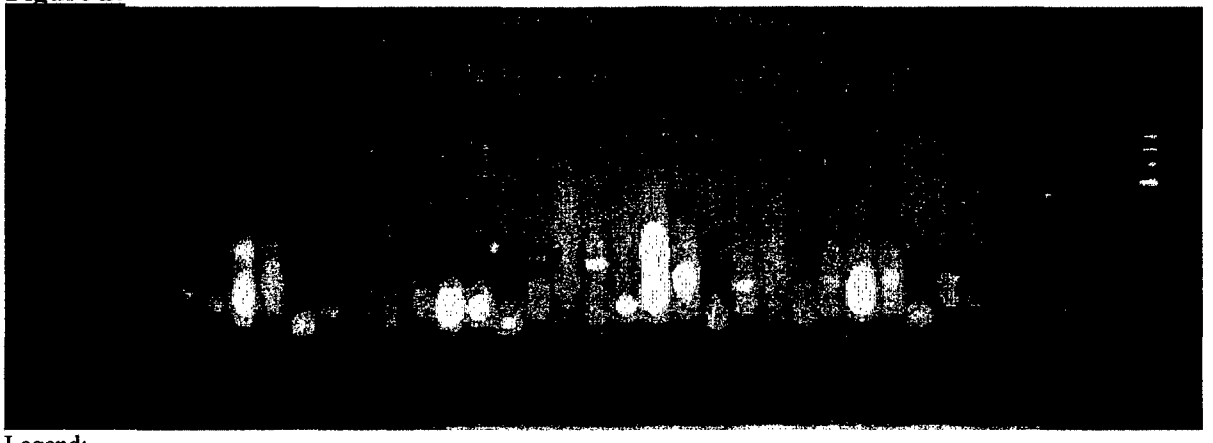
PCR. mRNA (1.0 μg) from preconfluent undifferentiated, spontaneously differentiated, and dexamethasone-induced differentiated 3T3-L1 cells was reverse transcribed into cDNA in 1x PCR buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 1 mM each of 4 dNTPs, 1 unit/μl RNasin inhibitor, 2.5 μM random hexamer primers, and 2.5 units/μl Moloney Leukemia Virus reverse transcriptase (**MuLV-RT**). Mixtures were annealed at room temperature for 10 min, incubated at 42°C for 40 min, heated at 95°C to inactivate MuLV-RT, and cooled for 5 min. Samples were amplified with promoter specific primers for 35 cycles (94°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec) with a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose/Tris-borate-EDTA gel (Fig. 1). We suspect the band visible at ~100 bp in lanes 4, 5, 8, 9 and 13 resulted from nonspecific primer annealing and amplification.

Preconfluent undifferentiated 3T3-L1 cells (lanes 6-9) expressed both promoter 1.3- and promoter II-specific aromatase mRNA. A similar pattern of promoter usage was reported in breast cancer tissue (Bulun et al., 1997; Harada, 1997; Zhou et al., 1996). Spontaneously differentiated cells (lanes 2-5) expressed only promoter 1.3-specific aromatase mRNA. In contrast, DEX-treated differentiated 3T3-L1 cells (lanes 10-13) expressed both promoter 1.3- and I.4-specific aromatase mRNA. Interestingly, with the I.4 specific primer set, two faint bands were observed in lane 12 (MW ~250 and ~400, respectively). Previous reports (Harada, 1992; Mahendroo et al., 1993) indicated that promoter I.4 was predominant in human adipose stromal cells and fibroblasts from normal breast tissue. Our preliminary findings suggested that spontaneously differentiated 3T3-L1 cells may not be equivalent to DEX-treated differentiated cells when used to investigate promoter-switching associated with breast tumorigenesis. More importantly, the possibility of biochemical heterogeneity and any attendant importance in human cell culture systems subjected to selected

differentiation treatments will be established to insure increased applicability of future findings. ***Task 1c):*** Currently underway with completion projected towards the end of December 2005.

Task 2a): Completed. Human normal breast fibroblast cells were cultured to confluence and either allowed to differentiate spontaneously (Control) over an additional 7 d period or subjected to one of four treatments: 1) MIX (i.e., 250 nM dexametahsone plus 0.5 mM 1-methyl-3-isobutyl-xanthine plus 10μg/ml insulin); 2) TZD (i.e., 2,4-Thiazolidinedione); 3) MCF7 (i.e., MCF7 tissue-conditioned medium); or 4) T47D (i.e., T47D tissue-conditioned medium) for 2 d followed by a 5 d spontaneous growth period. As described earlier, cells were lysed using TRIzol (Gibco) to extract RNA, which was then stored at -80°C while awaiting RT-PCR. RNA samples were subsequently reverse transcribed into cDNA in 1x PCR buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 1 mM each of 4 dNTPs, 1 unit/μl RNasin inhibitor, 2.5 μM random hexamer primers, and 2.5 units/μl Moloney Leukemia Virus reverse transcriptase (**MuLV-RT**). Mixtures were annealed at room temperature for 10 min, incubated at 42°C for 40 min, heated at 95°C to inactivate MuLV-RT, and cooled for 5 min. Samples were amplified with promoter specific primers for 35 cycles (94°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec) with a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 2% agarose/Tris-borate-EDTA gel (Fig. 2).

Figure 2:



Legend:

Lanes: 1) 100bp MW ladder. 2) -RT. 3) –TMP. 4) Control ARO. 5) Control PI4. 6) Control PI3. 7) Control PII. 8) Control GAPD. 9) Control DBC. 10) Control LRH1. 11) DMI ARO. 12) DMI PI4. 13) DMI PI3. 14) DMI PII. 15) DMI GAPD. 16) DMI DBC. 17) DMI LRH1. 18) TZD ARO. 19) TZD PI4. 20) TZD PI3. 21) TZD PII. 22) TZD GAPD. 23) TZD DBC. 24) TZD LRH1. 25) MCF7 ARO. 26) MCF7 PI4. 27) MCF7 PI3. 28) MCF7 PII. 29) MCF7 GAPD. 30) MCF7 DBC. 31) MCF7 LRH1. 32) T47D ARO. 33) T47D PI4. 34) T47D PI3. 35) T47D PII. 36) T47D GAPD. 37) T47D DBC. 38) T47D LRH1. 39) 100bp MW ladder.

-RT= devoid of reverse transcriptase; -TMP = devoid of RNA template; ARO = aromatase; PI4 = aromatase promoter I.4; PI3 = aromatase promoter I.3; PII = aromatase promoter pII; GAPD = glycerophosphate dehydrogenase; DBC = dibutyryl cAMP; LRH1 = liver receptor homologue 1;

Spontaneous differentiation as well as that occurring under TZD, MCF7 or T47D treatment was accompanied with increased aromatase expression. Interestingly, DMI treated cells did not exhibit a detectable aromatase signal. GAPD detected using enzymatic assays was established as a marker of pre-adipocyte terminal differentiation in 3T3-L1 cells (Kozak and Jensen, 1974; Wise and Green, 1979). During the course of our studies we have established and validated quantitation

of GAPD in human breast fibroblasts using end-point PCR. We are currently validating a quantitative real-time PCR enhancement of method. Results obtained from non-quantitative end-point PCR suggest GAPD was expressed in all differentiated cell preparations except in T47D treated cells. This finding coupled with the apparent lack of aromatase in DMI treated differentiated cells presents a challenge in arriving at an *in vitro* model that would be sufficiently representative of critical *in vivo* processes. Characterization of these alternate differentiation protocols is in progress.

Task 2 b): Experiments have been completed and samples are in storage awaiting assay and further analysis.
Tasks 3a and 3b): Experiments have been completed and samples are in storage awaiting assay and further analysis.
Tasks 3c, 3d and 3e): Currently underway and progressing as data is obtained on completion of ongoing sample assays.

Key Research Accomplishments

- Design, construction and validation of cDNA mimics.
- Design, construction and validation of primer-specific aromatase mRNA variants.
    - Amplification of invariant region encoded by exons II through X.
    - Amplification of individual aromatase species.
- Establishment and validation of murine cell-differentiation protocols as an initial template for human breast cell-differentiation.
- Design, construction and validation of primers for glycerophosphate dehydrogenase (GAPD) end-point PCR for assessment of *in vitro* human breast fibroblast differentiation.
- Establishment and ongoing validation of human breast fibroblast differentiation protocols currently undergoing validation.
-

Reportable Outcomes

- The following reportable outcomes are expected during the first quarter of 2006:
    - Two or more manuscripts
    - Two or more abstracts and presentation(s).
    - Two or more applications for substantial funding from national granting-making agencies.

Conclusions

Preliminary findings have confirmed the technical feasibility and soundness of key aspects of the proposed work. The process of identifying a cell differentiation protocol that would be most universally representative of critical *in vivo* processes is underway and expected to be completed in the next few months. That outcome is expected to significantly facilitate further exploration of aromatase promoter-switching and its role in breast cancer development and/or proliferation.

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Appendices

None